A Rapid Method of Purification of Leucine Dehydrogenase by Affinity Chromatography (Commemoration Issue Dedicated to Professor Yuzo Inouye on the Occasion of his Retirement)

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NOTE

A Rapid Method of Purification of Leucine Dehydrogenase by Affinity Chromatography

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Leucine dehydrogenase (L-Leucine: NAD^+ oxidoreductase, deaminating, EC 1.4.1.9) catalyzes the reversible deamination of L-lecuine and several other branched-chain and straight-chain L-amino acids to their keto analogs. We have purified the enzyme from *Bacillus sphaericus* to homogeneity by several steps and crystallized it, and studied the enzymological and physicochemical characteristics[1~3] the antineoplastic activity[4] and the stereo-structure by small-angle X-ray scattering studies.[5] We have reported also a simple and sensitive spectrophotometric method for the determination of branched-chain L-amino acids and their keto analogs with the enzyme, and its application to the assays of D-amino acid amino-transferase[6] and leucine aminopeptidase.[7] The spectrophotometric method with leucine dehydrogenase was shown to be useful to the determination of branched-chain L-amino acids and keto acids in rat tissues.[8] Recently, Hummel *et al.* suggested that the enzyme can be applied to the production of branched-chain L-amino acids[9]; the previous purification method of the enzyme was time-consuming and tidious. Thus, the convinient procedure for purification of the enzyme is required. We here report a very simple and effective purification method of the enzyme from cells of *B. sphaericus* using 5′-AMP-Sepharose 4B affinity chromatography.

Leucine dehydrogenase activity was assayed essentially as previously described[2] by following the reduction of NAD^+ except that the final volume of the reaction mixture was 1.0 ml. Protein concentrations were determined by the methods of Lowry *et al.*[11], and of Kalb and Baernlohr.[10] However, bovine sereum albumin (Sigma Chemical Co.) was used as a standard protein instead of egg albumin (Nakarai Chemicals) which was used in the previous study.[2] B. *sphaericus* (IFO 3525) was cultured with the 600-ml medium placed in a 2-liter flask on a reciprocating shaker at 30°C for 18 h. The harvested cells were washed with 0.85% NaCl and subsequently with 0.01 M potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercap-

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toethanol. This buffer system was used throughout the study. All purification operations were performed at 0–5°C, unless otherwise stated.

The cells (wet weight: about 12 g) were disrupted with aluminium oxide and then suspended in an adequate volume of the buffer. Intact cells, cell debris and aluminium oxide were removed by centrifugation at 10,000×g for 20 min. The supernatant solution (crude extract) was brought to 70% saturation with $(NH_4)_2SO_4$, and the suspension was applied to a column (3×9.5 cm) of cellulose (cellulose powder CF from whatman, England), previously equilibrated with the buffer containing the same concentration of $(NH_4)_2SO_4$. The pH was previously adjusted to 7.2 with 14% NH_4OH. After washing the column with the same buffer solution, a large amount of impurities were eluted with the buffer containing 40% saturated $(NH_4)_2SO_4$ as shown in Fig. 1. The enzyme was eluted with the buffer containing 20% saturated $(NH_4)_2SO_4$. The active fractions were pooled, and concentrated by addition of solid $(NH_4)_2SO_4$ (50% saturation) and centrifugation. The precipitate was dialyzed against the buffer, and the enzyme solution (15 ml) was loaded onto a DEAE-cellulose column (1.7×18 cm) equilibrated with the same buffer. The column was successively washed with the buffer and the same buffer containing 0.2 M NaCl, and then the enzyme was eluted with the buffer containing 0.35 M NaCl. The enzyme solution was concentrated with an ultrafiltration membrane (UK–10, Toyo Roshi Co., Tokyo), and then dialyzed against 0.01 M potassium phosphate buffer (pH 6.5) containing 0.01% 2-mercaptoethanol. 5′-AMP-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala), which is a group specific adsorbent for separating NAD^+-dependent dehydrogenases and kinases, was used for the further purification of the enzyme. Potassium phosphate (0.01 M, pH 6.5) was used for this affinity chromatography since leucine dehydrogenase was adsorbed by the adsorbent more effectively at the acid pH than at the neutral one. The enzyme solution (28 ml) was applied to a 5′-AMP-Sepharose 4B column (1.6×7 cm) equilibrated with the above buffer. After the column was washed with the buffer and the same buffer containing 0.1 M NaCl, the enzyme was eluted with the same buffer

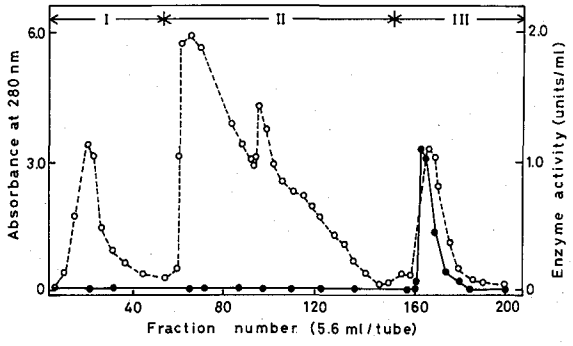

Fig. 1. Negative Salting Out-Chromatography with Ammonium Sulfate
The concentrations of $(NH_4)_2SO_4$ in potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercaptoethanol were: I, 70% saturation; II, 40% saturation; and III, 20% saturation. ○·····○, Absrobance at 280 nm and ●——●, leucine dehydrogenase activity.

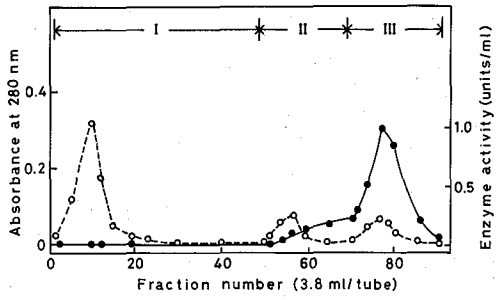

Fig. 2. 5′-AMP-Sepharose 4B Affinity Chromatography
The elution buffers used were: I, potassium phosphate buffer (pH 6.5) containing 0.01% 2-mercaptoethanol; II, the same buffer containing 0.1 M NaCl; III, the same buffer containing 0.35 M NaCl. ○·····○ Absorbance at 280 nm and ●——● leucine dehydrogenase activity.

supplemented with 0.3 M NaCl (Fig. 2). The active fractions were pooled and concentrated by ultrafiltration. Polyacrylamide gel (7.5%) electrophoresis of the enzyme preparation obtained showed two protein bands, and only a major band was visualized by activity staining.[12] To eliminate the containant protein, the gel permiation chromatography on a Sephadex G-150 column (2.2×85 cm) was employed. The enzyme solution (8 ml) was applied to the column equlibrated with 0.01 M potassium phosphate buffer (pH 7.2), and then eluted with the same buffer. The activefractions were collected and concentrated. The purified enzyme preparation was found homogeneous upon polyacrylamide gel electrophoresis. A protocol of the purification is presented in Table I. When the enzyme activity was estimated with bovine serum albumin as a standard, the specific activity of homogeneous preparation was about 25, which is approximately 60% of the value calculated with egg albumin as described previously.[2] This improved method can make us to save time for the purification of leucine dehydrogenase.

Table I. Summary of purification of leucine dehydrogenase

Steps	Total protein	Total activity	Specific activity	Yield	Purification
	(mg)	(units)	(units/mg)	(%)	(fold)
Crude extract	1660	181	0.11	100	1
Negative salting out with $(NH_4)_2SO_4$	242	102	0.42	56	3.8
DEAE-cellulose	15	70	4.7	39	43
5′-AMP-Sepharose 4B	1.5	37	20	18	182
Sephadex G–150	0.73	18	25	10	227

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